

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
 - TEXT CUT OFF AT TOP, BOTTOM OR SIDES
 - FADED TEXT
 - ILLEGIBLE TEXT
 - SKEWED/SLANTED IMAGES
 - COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
 - GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 5/06, 5/10, A61K 48/00 C12Q 1/02	A1	(11) International Publication Number: WO 94/09118 (43) International Publication Date: 28 April 1994 (28.04.94)
(21) International Application Number: PCT/US93/09718 (22) International Filing Date: 13 October 1993 (13.10.93) (30) Priority data: 07/959,842 13 October 1992 (13.10.92) US (60) Parent Application or Grant (63) Related by Continuation US 07/959,842 (CIP) Filed on 13 October 1992 (13.10.92) (71) Applicant (for all designated States except US): THE GENERAL HOSPITAL CORPORATION [US/US]; 55 Fruit Street, Boston, MA 02114 (US).		(72) Inventor; and (75) Inventor/Applicant (for US only) : GOLDRING, Mary [US/US]; 30 Vista Avenue, Auburndale, MA 02166 (US). (74) Agent: CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US). (81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: IMMORTALIZED HUMAN CHONDROCYTES (57) Abstract In the field of chondrocyte culture and biology related to using chondrocytes for screening and treatment of cartilaginous diseases, a problem has existed in obtaining immortalized chondrocytes that retain their differentiated characteristics. A method of immortalizing chondrocytes so that they retain their differentiated characteristics and the cells so immortalized are disclosed. Methods of using the cells in gene therapy approaches to cartilaginous disease and methods of using the cells to screen for agents that affect chondrocyte function are disclosed. The use of chondrocytes to produce agents that inhibit vascularization is also disclosed.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LJ	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TG	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

- 1 -

IMMORTALIZED HUMAN CHONDROCYTES

This invention relates to the field of chondrocytes.

5 Background of the Invention

The chondrocyte is a terminally differentiated cell that functions to maintain the cartilage specific matrix phenotype under normal conditions of low turnover. The chondrocyte is derived from the chondroblast, a
10 mesenchymal derivative. At the cellular level cartilage tissue is composed only of chondrocytes and does not contain nerves, blood vessels or lymphatics. The cartilage tissue is capable of both a positional and interstitial growth which occurs by cell division of the
15 mature chondrocyte and the secretion of an extracellular matrix. The major constituents of the extracellular matrix of mature hyaline cartilage found in diarthroidal joints are type II (with some types IX and XI) collagen and the cartilage-specific, large aggregating
20 proteoglycan.

High oxygen tension, in general, blocks cartilage growth and vascularization and mineralization around cartilaginous tissue are found to be inhibited. It is thought that this inhibition occurs by the secretion of
25 specific inhibitors from chondrocytes and the isolation of such inhibitors is a source of interest in current chondrocyte research.

Under pathological conditions, i.e., inflammatory joint disorders, chondrocyte function is altered and
30 there are changes in the composition of the extracellular cartilage matrix (Krane et al., 1986, In: Articular Cartilage Biochemistry, Kuettner et al., eds., Raven Press, New York, p413; Dodge and Poole, 1989, Journal Clinical Invest. 83:647; Hamerman, 1989, New England
35 Journal of Medicine 320: 1322; Harris, 1990, N. Engl. J.

- 2 -

Med. 322:1277). In conditions such as rheumatoid arthritis and osteoarthritis, changes in the production and temporal release of cytokines result in altered chondrocyte function. This altered function contributes to the erosion and inappropriate repair of cartilage matrix (Krane et al., 1988, In: Cell and Molecular Biology of Vertebrate Hard Tissues. Ciba Foundation Symposium 136. Evered and Harnett, eds., Wiley, Chichester, p. 239; Krane et al., 1990, Annals NY Acad. Sci. 580:340).

Mature differentiated chondrocytes can be defined by numerous cellular characteristics: the production of predominately type II collagen, the secretion of a specific profile of proteoglycans, the production of link protein and specific cellular responses to the cytokines interleukin-1 (IL-1) and interferon- γ (IFN- γ) (Goldring and Goldring, 1990, Clinical Orthopedics and Related Research).

The generation of immortalized chondrocyte cell lines has been pursued in numerous animal systems (Horton et al., 1988, Experimental Cell Res. 178:457; Grigoriadis, et al., 1988, J. Cell Biol. 106:2139; Thenet, et al., 1992, J. Cell Physiol. 150:158; Gionti, et al., 1985, Cell Biol. 82:2756; Takigawa, et al., 1989, Cancer Res. 49:3996; Bernier, et al., 1990, J. Cell. Physiol. 145:274; Takigawa, et al., 1991, Int. J. Cancer 48:717). Such efforts have yielded immortalized chondrocyte derived cell lines which exhibit some of the aforementioned characteristics often, if not always, present in a truly differentiated cell line. In contrast to mature primary chondrocytes in culture, many of the immortalized chondrocyte derivatives display some fibroblastic phenotypes indicative of only partial differentiation. Immortalized cell lines and methods for obtaining such cell lines which display most, if not all,

- 3 -

the major phenotypes and profiles of gene regulation of a differentiated chondrocyte would be of use in a variety of research and clinical applications.

Summary of the Invention

5 In general, the invention provides an immortalized human chondrocyte cell line having a homogeneous polygonal morphology which, in addition, is further characterized in that it expresses type II collagen, suppresses the expression of type II collagen in response
10 to IL-1 and IFN- γ , expresses collagenase in response to IL-1, and expresses the proteoglycans expressed by human primary chondrocytes, but does not express the proteoglycan Versican not normally found in cartilage. In addition, the invention provides an immortalized
15 chondrocyte cell line in insulin-containing serum substitute characterized in that it has the same gene expression profile characterized above and corresponding to that of mature primary chondrocytes in culture.

 The invention further includes any immortalized
20 cell line with the aforementioned characteristics derived from human primary chondrocytes transfected with origin defective SV40.

 The aforementioned immortalized human chondrocyte cell lines are made by a procedure which involves
25 isolating human chondrocytes, preferably juvenile primary chondrocytes, and growing them in culture, transfecting the culture with origin-defective SV40 or another immortalizing nucleotide sequence, subculturing the transfected cells, preferably for a period of greater
30 than six weeks, and selecting a cell line that displays a cobblestone morphology and is further characterized in that it a) expresses type II collagen, b) produces less of type II collagen in response to IL-1 and IFN- γ , c) expresses collagenase in response to IL-1, d) secretes a

- 4 -

proteoglycan profile corresponding to that of mature human chondrocytes. The cell line may be maintained in medium with insulin-containing serum, for example, as Nutridoma-SP to obtain enhanced expression of primary
5 chondrocyte phenotypes. Preferably, the phenotypic selection for immortalized chondrocytes is done following a drug resistance selection which eliminates those cells which do not bear the transfected nucleic acids.

The invention also provides a method of treating a
10 degenerative cartilage disease by transfecting cells obtained from a cell line described above with a gene encoding a therapeutic protein, and administering the transfected cells to the patient. This method may be used to treat the degenerative cartilage disease
15 osteoarthritis. The transfection method may also be used to incorporate a gene encoding a cytokine, preferably TGF- β and/or IGF-I to treat diseases of the cartilage, for example diseases of cartilage degradation.

Transfection may also be used as stated above to
20 incorporate a gene encoding a cytokine inhibitor such as IL-1, a similarly acting receptor antagonist, or an IL-1 mutant. The same method of transfection may also be used to transfect genes encoding proteases or protease inhibitors, or extracellular matrix components.
25 Immortalized chondrocytes containing a gene encoding a therapeutic protein may be administered to the patient by localized injection.

The invention also provides a method by which the immortalized chondrocytes described above may be used as
30 a source for harvesting antivasicular compounds by a method involving culturing the immortalized chondrocyte cells and purifying from these cells a compound which prevents vascularization.

The invention further provides a method of
35 screening for compounds useful in the therapy of

- 5 -

cartilaginous diseases. This method involves applying potential therapeutic compounds to the immortalized chondrocyte cells and then monitoring the cells for alterations in gene regulation which ameliorate the pathological condition.

Other features and advantages of the invention will be apparent from the following detailed description and other embodiments of the invention and from the claims.

10

Detailed Description

The drawings will first be briefly described.

Drawings

Fig. 1A is a photograph of the T/C-28a2 cells in culture and Fig. 1B a photograph of the C20/A4 cell line in culture.

Fig. 2 depicts the expression of type II collagen mRNA in the immortalized chondrocyte cell line C20/A-4.

Fig. 3A and Fig. 3B depict the expression of type II collagen mRNA in the immortalized chondrocyte cell line T/C-28a2 in a variety of media. The first 5 lanes of Fig. 3A represent type II mRNA expression in primary chondrocytes. Lanes 6-24 of Fig. 3A represent the type II mRNA levels in T/C-28a2 in a variety of media. The autoradiograph shown in Fig. 3A is of a brief exposure of the Northern blot, and Fig. 3B is a longer exposure of the same blot depicted in Fig. 3A.

Fig. 4 is a graph showing the detection of expression of the proteoglycans aggrecan, PGI, PGII and versican in addition to collagen IX in diverse media for both normal and immortalized T/C-28a2 chondrocytes.

Fig. 5 depicts the expression of collagenase and early gene mRNAs in response to IL-1 in human costal chondrocytes at 3 hours, 6 hours and 24 hours.

- 6 -

Fig. 6 depicts the level of c-Jun mRNA expression at 3 and 5 hours after the addition of IL-1 β in C20-A4 cells.

Fig. 7 depicts primary costal chondrocytes (day 11 of primary culture) which were preincubated for 24 h alone (control) or with hIL-1 β (5 pM) without or with indomethacin (1 μ M).

Fig. 8A and Fig. 8B depict costal chondrocytes (see Fig. 7) which were preincubated for 24 h alone (control) or with IFN- γ (10, 100, and 500 units/ml).

Fig. 9 represents the dot hybridization analysis of procollagen mRNA levels in costal chondrocytes treated with IFN- γ and IL-1 β .

Fig. 10 depicts the kinetics of α 1(II) and α 1(I) procollagen mRNA expression in costal chondrocytes treated with IL-1 β and indomethacin in the absence and presence of cycloheximide.

Fig. 11 is a set of graphs which represent the analysis of the stability of α 1(II) and α 1(I) procollagen mRNAs treated with IL-1 β in the absence and presence of cycloheximide (CHX).

Figs. 12A and 12B show the expression of 5'-flanking regulatory sequences of COL2A1 by human costal chondrocytes in different culture conditions.

Fig. 13 depicts the dose-dependent inhibition of expression of pCAT-B/4.0 by IL-1 β and IL-1 α .

Fig. 14 depicts the dose-dependent suppression of pCAT-B/4.0 (PCAT4.0) expression and potentiation of this suppression by indomethacin in human costal chondrocytes.

Fig. 15 shows the expression of pCAT-B/4.0 in rat chondroblasts and inhibition by human IL-1 β and murine IFN- γ .

Fig. 16 is a graph demonstrating the expression of pCAT Control, but not pCAT-B/4.0, by human dermal fibroblasts.

- 7 -

Fig. 17A is a table representing the CAT activity of plasmids pCAT4.0, pCAT2.9 and pCAT2.0 in both control and hIL-1 β serum conditions. Fig. 17B depicts the effect of recombinant IL-1 β on expression of COL2A1 regulatory sequences in immortalized human chondrocytes. 17(C) depicts the effect of recombinant IFN- γ on expression of COL2A1 regulatory sequences in immortalized human chondrocytes.

The following examples are provided to illustrate the advantageous characteristics and methods of making and testing the immortalized chondrocytes of the invention; these examples are not intended to limit the invention.

I. Generation of Immortalized Human Chondrocyte Cell

15 Lines

A. Isolation of Primary Chondrocytes.

Human costal cartilage was obtained from ribs removed during pectus excavatum repair of a 5-year-old juvenile male and a 15-year-old female. Chondrocytes were isolated by dispersion with proteases and cultured as described below. Cartilage slices were incubated at 37°C with hyaluronidase (1mg/ml in PBS; bovine testicular from Sigma) for 10 min, followed by 0.25% trypsin (GIBCO/BRL) for 45 min. The cartilage was then chopped in small fragments and incubated at 37°C with collagenase (3 mg/ml in serum-free culture medium; clostridial peptidase from Worthington) for 48 h. The dispersed cells were then washed with Ca⁺⁺- and Mg⁺⁺-free PBS and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) with medium changes every 3-4 days, as described previously (Goldring and Krane, 1987, J. Biol. Chem. 262:16724; Goldring et al., 1988, J. Clin. Invest. 82:2026).

- 8 -

B. Isolation of the C-20/A4 Cell Line.

An immortalized human chondrocyte cell line was generated using pMK/SV40 ori⁻ plasmid transfection and is termed C-20/A4. C-20/A4 was derived from juvenile costal
5 chondrocytes isolated from a specimen of rib cartilage from a 5-year-old male obtained as discarded material after surgery as described above. At day 10 of primary culture 10 confluent 75-cm² flasks were transfected with origin-defective SV40 (20 µg/flask of pMK/SV40 ori⁻
10 plasmid DNA) (Small et al., 1982, Nature 298:671) using the polybrene/DMSO method (Chaney et al., 1986, Somatic Cell Molec. Genet. 12:237). The immortalized cells were obtained by the selection of foci 4-6 weeks after transfection and continuous subculture to produce
15 senescence of the parental cells. Of the 140 foci (10-20 per flask) selected originally, only 20 were subcultured. After 20-40 passages, the C-20/A4 line was selected for further characterization, since these cells continued to grow well and maintain a homogenous polygonal morphology
20 characteristic of chondrocytes rather than fibroblasts. Growth in a insulin-containing media such as Nutridoma-SP (Boehringer Mannheim) was found to be required for the expression of the full range of primary chondrocyte phenotypes by the immortalized chondrocytes in culture.
25 Figure 1B depicts the C-20/A4 cells in culture.

The C-20/A4 cells have been maintained in culture for over three years, during which time they have continued to grow well and maintain a homogenous polygonal morphology. Additional immortalized cell lines
30 with characteristics similar to those of C-20/A4 may be obtained from the 120 immortalized clones or by replicating the immortalization procedure.

C. Isolation of T/-28 Cell Lines.

The SV-40 large T-antigen encoding DNA was
35 inserted in the pZip Neo SV(X) vector (Psi-2 U19-5

- 9 -

plasmid) (Williams et al., Mol. Cell Biol. 8:3864) and freshly isolated cultures of juvenile human costal chondrocytes from a specimen of rib cartilage from a 15-year-old female obtained as discarded material after surgery (pectus excavatum repair) at day 5 of primary culture were used for immortalization. Cells were cultured for more than 8 weeks before the initial G418 drug resistance selection. After selection of transfected cells in G418, the uncloned cell populations (designated T/C-28) were established through several subcultures following G418 selection. Cells were then further selected by limited dilution or by plating on agarose and then transferring back to monolayer culture on plastic for cloning by limiting dilution. Over 40 lines were established, at least 75% of which had the polygonal or cobblestone morphology characteristic of chondrocytes. Growth in a insulin-containing media such as Nutridoma-SP (Boehringer Mannheim) was found to be required for the expression of the full range of primary chondrocyte phenotypes by immortalized chondrocytes in culture. Figure 1A depicts the T/C-28a2 cell line in culture.

Generation of these stable T/C-28 chondrocyte lines was accomplished most readily after further selection of neomycin-resistant cells by agarose suspension culture for 2 to 4 weeks, as described for chick embryo chondrocytes by Castagnola et al. (J. Cell Biol. 102:2310-2317, 1986). For example, the non-cloned T/C-28 chondrocytes at passage 20 were incubated with trypsin-EDTA and the resulting cell suspension was washed and transferred to plates that had been coated with 1% agarose. These chondrocytes aggregated and began within 24 h to form clumps that increased in size and number through the first week, as we have observed in agarose suspension cultures of normal human chondrocytes. After

- 10 -

10 to 14 days of agarose culture, the clumps began to disaggregate, become smaller and form a single cell suspension. When this suspension was replated in tissue culture dishes, a more homogeneous monolayer of polygonal cells was generated that proliferated readily in culture medium containing 10% FCS and reached confluence within one week. Confluent cultures formed nodules reminiscent of the original transformed foci. These agarose-selected cells, passaged weekly at subconfluence, have maintained stable morphology and proliferative capacity throughout a further 40 passages and have been used in the studies described below. Further cloning by limited dilution of the agarose-selected cells was easily accomplished directly after replating the cells on tissue culture plastic. Characteristics of both the uncloned population selected on agarose (T/C-28a2) and several of the cloned lines (e.g., I-2 and D-8 clones) are described below.

Both the C-20/A4 and T/C-28 chondrocyte lines have retained the capacity to produce immunoreactive T antigen throughout the various selection procedures.

The T/C-28a2 cell line derived from the T/C-28 population, as well as other similar lines, has maintained its chondrocyte phenotype for over 40 passages in culture. Lines with characteristics similar to T/C-28a2 may be isolated from the T/C-28 population or from primary chondrocytes using the described methods.

II. Characterization of Chondrocytes.

A. Overview of expression of cartilage-specific phenotypes by immortalized human chondrocytes.

Stable proliferating lines of immortalized human chondrocytes have been generated, as described above, that express differentiated cartilage-specific phenotypes under defined conditions. Type II collagen synthesis with minimal type I collagen was observed in the agarose-

- 11 -

selected T/C-28 chondrocytes even in the presence of 10% FCS, but in cells continuously cultured in monolayer, type II collagen expression was best when the concentration of FCS was reduced to 0.5%. 10% FCS

5 suppresses the capacity of the normal human chondrocytes to express regulatory sequences of the type II collagen gene in transient transfection experiments and removal of serum and addition of an insulin-containing serum substitute is permissive for expression of either

10 endogenous type II collagen or the transfected type II collagen gene regulatory sequences even in subcultured chondrocytes. The immortalized chondrocytes display these features as well; the C-20/A4 and T/C-28 chondrocyte lines can be grown and subcultured

15 continuously in culture medium containing 10% FCS, and they exhibit high expression of type II collagen mRNA after one to several days of culture in serum-free defined medium containing 1% Nutridoma-SP (Boehringer-Mannheim). Expression of other cartilage-specific

20 collagen mRNAs, including those for type IX and type XI can also be observed under these conditions and addition of IGF-I and/or TGF- β may permit optimal expression of type II collagen mRNA. These immortalized cells also express cartilage-specific aggrecan mRNA, as well as the

25 small proteoglycan biglycan and decorin mRNAs appropriately. Expression of mRNA encoding versican, a small proteoglycan not found in cartilage, has not been observed under any condition of culture.

Immunocytochemical analyses reveals that the T/C-

30 28 chondrocytes synthesize and deposit in the extracellular matrix proteins immunoreactive with anti-proteoglycan (chondroitin-4-sulfate and large proteoglycan, from Seikagaku, Tokyo, Japan) and type II collagen (from Chemicon, Temecula, CA) monoclonal

35 antibodies. When the cells were plated on the chamber

- 12 -

slides and maintained in culture medium containing 10% FCS, no immunoreactive type II collagen or proteoglycan could be demonstrated. When the FCS was removed and 1% Nutridoma was added for 1 to 3 days, type II collagen and proteoglycan immunoreactivity was observed. When ascorbate was added to the cultures during the final incubation period with Nutridoma, both type II collagen and proteoglycan were deposited in the matrix. In the absence of ascorbate, the type II collagen immunoreactivity remained within the cells and the staining for proteoglycan was punctate rather than diffuse in the extracellular matrix. Staining for the nonspecific chondroitin-0-sulfate was not observed under any condition.

15 A comparison of the characteristics of representative immortalized human chondrocyte cell lines and primary human chondrocyte cell lines is shown in Table 1.

20 B. Probes used to characterize immortalized human chondrocytes.

Collagen Probes.

Synthesis of cartilage specific collagens (types II, IX and XI collagens) may be detected versus the following probes: Type II collagen mRNA may be detected using the hgColII probe, which is a 3.8 kb genomic fragment of the human $\alpha 1(\text{II})$ procollagen gene encoding amino acids 892 to the end of the C-propeptide (-1150 nucleotides of exon material) (Sandell, L.J. J. Cell Biol. 99:405, 1984; Goldring et al., J. Biol. Chem. 261: 9049-9055, 1986; Goldring et al., J. Clin. Invest. 82: 2026-2037, 1988; Sandell et al., Conn. Tiss. Res. 17: 11-22, 1988); or the pkTh1330 probe, which is a -600-bp fragment encoding a helical portion of the type II collagen chain near the C-propeptide and was cloned using a human costal chondrocyte library available from Dr. T.

- 13 -

Kimura, Osaka, Japan and Dr. M. Goldring, Boston, MA); also useful is pCAT-B/4.0, which includes the 5'-flanking regulatory sequences (-577 to +3428) of the human type II collagen gene including the promoter and an enhancer in
5 the first intron (Ryan et al., Genomics 8: 41-48, 1990; Goldring et al., Arthritis Rheum. 34: S106, 1991; and Sandell et al., Orthopaed. Res. Trans. 17: 1, 1992b.

Type IX collagen mRNA may be detected using the pkTh123 probe which includes 600 bp cDNA encoding a
10 portion of the human $\alpha 1(\text{IX})$ procollagen subunit, prepared using a cDNA library prepared from a human costal chondrocyte mRNA (Goldring et al., J. Clin. Invest. 82: 2026-2037, 1988; Kimura et al., Eur. J. Biochem. 179: 71-78, 1989).

15 Type XI collagen transcription may be detected using the pkTh181 probe which is a 1265 bp cDNA encoding a helical portion of the human $\alpha 2(\text{XI})$ procollagen subunit (Kimura et al., J. Biol. Chem. 264: 13910-13916, 1989).

Collagens which are not present in articular
20 cartilage but associated with chondrogenesis and/or growth plates include Type X and Type IIA collagens. Type X collagen probes are used to assess chondrogenic potential of cloned lines (Sandell et al., Orthopaed. Res. Trans. 17: 1992)) and Type IIA probes which reveal
25 alternatively spliced type II collagen mRNA may also be used (Sandell et al., J. Cell Biol 114: 1307-1319, 1991; Sandell et al., Articular Cartilage and Osteoarthritis, 1992).

Other collagens of importance in characterizing
30 potential immortalized chondrocyte lines include Type I collagen and Type III collagen, since they are detected only in dedifferentiated chondrocytes. Transcription from the Type I collagen gene may be detected using the Hf677 probe which is a 1500 bp cDNA encoding part of the
35 $\alpha 1(\text{I})$ procollagen subunit (Chu et al., Nucleic Acids Res.

- 14 -

10: 5925-5934, 1982; Goldring et al., J. Biol. Chem. 262: 16724-16729, 1987; Goldring et al., 1988); or Hf1131 which is a 1500 bp cDNA probe encoding part of the $\alpha 2(I)$ procollagen subunit (Bernard et al., Biochemistry 22: 5 1139-1145, 1983; Goldring et al., J. Biol. Chem. 262: 16724-16729, 1987; Goldring et al., J. Clin. Invest. 82: 2026-2037, 1988). Transcription from the type III collagen gene is detected using the pHC III-1 probe, a 1885 bp cDNA encoding the C-propeptide and portions of 10 the helical and untranslated regions of $\alpha 1(III)$ procollagen (Miskulin et al., Biochemistry 25: 1408-1413, 1986; Goldring et al., J. Biol. Chem., 262: 16724-16729, 1987; Goldring et al., J. Clin Invest., 82: 2026-2037, 1988).

15 Collagens may also be detected using immunoreactive antibodies which are readily available.

Proteoglycan Probes.

The following probes are discussed in Hering et al., Orthopaed. Res. Trans. 17: 682, 1992 and can be used 20 to monitor the synthesis of proteoglycans. Cartilage-specific proteoglycan synthesis may be detected using either nucleic acid probes or specific immunoreactive antibodies to Aggrecan (CSPG, large aggregating proteoglycan), Link protein, Biglycan (PG-I, also called 25 PG-40), and Decorin (PG-II).

No cartilage-specific proteoglycans may be detected using Versican.

House-keeping gene probes.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 30 cDNA probe may be used to monitor the general status of transcription of genes required for cellular maintenance functions (MacNaul et al., J. Biol. Chem., 265: 17238-17245, 1990).

Proteinase and proteinase inhibitor probes to 35 detect chondrocyte cell line responses to cytokines.

- 15 -

- Collagenase gene expression is monitored using XHF₁ which is a cDNA probe for human procollagenase which is a 1.5 kb XbaI cDNA fragment (Stephenson et al., Biochem. Biophys. Res. Commun. 144: 583-590, 1987;
- 5 MacNaul et al., J. Biol. Chem., 265: 17238-17245, 1990). Stromelysin gene expression is monitored using a 1.8 kb PstI cDNA fragment (MacNaul et al., J. Biol. Chem., 265: 17238-17245, 1990). Tissue inhibitor of metalloproteinases (TIMP) is monitored using 0.9 kb
- 10 HincII cDNA fragment (MacNaul et al., J. Biol. Chem., 265: 17238-17245, 1990). The above three cDNA probes are provided by Dr. Nancy I. Hutchinson, Merck, Sharp & Dohme Research Laboratories, Rahway, NJ. Plasminogen activator and PA inhibitor probes, are also generally available.
- 15 **Transcription factor probes.** c-jun transcription was monitored using a cDNA (pH540) encoding human c-Jun (Nakabeppu et al., Cell 55: 907-915, 1988); Jun B transcription was monitored using a 1800 bp cDNA (p465.20) encoding mouse Jun B (Ryder et al., Proc. Natl. Acad. Sci. USA 85: 1487-1491, 1988); Egr-1 transcription
- 20 was monitored using a 3.1 kb EcoRI cDNA fragment (OC3.1) (Sukhatme et al., Cell 53: 37-43, 1988); and c-fos was monitored using a cDNA provided by Dr. R. Tijian, Berkeley, CA (Rauscher et al., Science, 240: 1011-1016, 25 1988).

Other available probes.

The following probes are available for the characterization of chondrocytes.

- For assessment of osteogenic potential the
- 30 following probes may be used: hon-164 (human osteonectin (0.5 kb); hon-2 (human osteonectin (1.9kb, for riboprobe)); OP-10 (human osteopontin); BG-5g (human BSP-II); p16 (human PG-I (biglycan); p2 (human PG-II (decorin, PG-40); pSP65 (human osteocalcin); and a human
- 35 calcitonin receptor probe is also available. These

- 16 -

probes were obtained from Drs. Marian Young and Larry Fisher, National Institutes of Health, Bethesda, MD, in collaboration with Dr. S. Goldring, Massachusetts General Hospital, Boston, MA.

- 5 For the assessment of basement membrane-specific mRNAs the following probes may be used: p1236 (human laminin B1); p1237 (human laminin B2); and p1239 (cDNA encoding $\alpha 2$ (IV) collagen chain of human type IV collagen). These probes may be used to rule out
10 endothelial cell contamination and were obtained from Dr. Y. Yamada in collaboration with Dr. S. Goldring, Massachusetts General Hospital, Boston, MA.

- For the assessment of cytokine mRNAs the following probes may be used: p3ACSFRI (human M-CSF); probes for
15 human GM-CSF; probes for human G-CSF; pXM209 (human IL-6); and pXMT2 (human IL-7). These probes are available from Dr. S. Clark, Genetics Institute, Andover, MA and Dr. Steven Goldring, Massachusetts General Hospital, Boston, MA). In addition, human IL-1 α and IL-1 β probes available
20 from Dr. P. Auron may be used for the identification of IL-1 gene expression.

C. Immortalized human chondrocytes express type II, type IX and type XI collagen mRNA.

- The C-20/A4 line expressed chondrocyte-specific
25 type II collagen mRNA when the cells were cultured in the presence of Nutridoma (Figure 2). The probe used to detect the presence of type II collagen RNA was the $\alpha 1$ (II) procollagen cDNA probe (pkTh1330) (Kimura et al., 1989, Eur. J. Biochem. 179:71). The cell line T/C-28a2
30 also expresses type II collagen mRNA (Fig. 3A and 3B).

- Analysis of RNA was done in the following manner: Cytoplasmic RNA for dot blots was prepared by the method of White and Bancroft (1982, J. Biol. Chem 257:8569) and as described previously (Goldring and Krane, 1987, supra;
35 Goldring et al., 1988, supra; and Goldring et al., 1986,

- 17 -

J. Biol. Chem. 261:9049). Total RNA for Northern blots was extracted according to a method adapted from Chomczynski and Sacchi (Anal Biochem, 1987, 162:156). Chondrocytes ($>1 \times 10^6$) from 6- or 10-cm plates were

5 lyzed directly in 500 μ l (final volume) of Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1M 2-mercaptoethanol) and sheared with a 27-gauge needle either before or after freezing at -80°C . The RNA was then extracted at 4°C by sequential addition

10 of 2 M sodium acetate (50 μ l), pH 4.0, water saturated phenol (500 μ l), and chloroform/isoamyl alcohol (49:1; 100 μ l) and precipitation of the aqueous phase with an equal volume of isopropanol. The RNA pellets were dissolved in 50 μ l of DEP-treated 0.01 M HEPES.

15 Alternatively, total RNA from $>10 \times 10^6$ cells was extracted as described by Sandell and Daniel (supra). The final preparations gave yields of approximately 10 μ g of RNA per 1×10^6 cells with the appropriate $A_{260}:A_{280}$ ratio of approximately 2.0. Northern blots were prepared

20 on either nitrocellulose or BAS-85 membranes (Schleicher & Schuell) as described previously (Goldring and Krane, 1987, supra; Goldring et al., 1988, supra; and Goldring et al., 1986, supra). Staining of 18S and 28S RNA bands in agarose gels with ethidium bromide and hybridization

25 of Northern blots with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (MacNaul et al., 1990, J. Biol. Chem. 265:17238) were employed to monitor uniform loading of RNA on Northern blots. Labeling of probes by nick translation and hybridization were

30 performed as described (Stephenson et al., 1987, Biochem. Biophys. Res. Commun. 144:583; Goldring and Krane, 1987, supra; Goldring et al., 1988, supra).

Expression of type II collagen is a hallmark of mature differentiated chondrocytes. The finding that the

35 C-20/A4 on T/C-28a2 cell lines express type II collagen

- 18 -

mRNA at levels comparable to mature primary chondrocytes provides strong evidence that these cell lines have maintained the characteristics of differentiated primary chondrocytes.

5 The pKTh123 and pKTh181 probes were used to monitor the expression of type IX and type XI collagen gene expression, respectively, in immortalized human chondrocytes. Using techniques analogous to those used to monitor type II expression, above, it was found that
10 both type IX and type XI collagens are expressed appropriately in immortalized chondrocytes, e.g., the expression profile is similar to that which is observed in primary human chondrocytes under similar conditions.

D. Transcription of Collagenase mRNA and early
15 genes is stimulated by IL-1 in both primary human chondrocytes and immortalized human chondrocytes.

Interleukin-1, a product predominantly of monocytes, increases the synthesis and release of procollagenase and prostaglandin E₂ by mesenchymal target
20 cells such as synovial fibroblasts and articular chondrocytes, an effect mimicked by some phorbol esters.

i) Primary chondrocyte responses.

Primary cultures of human articular chondrocytes were preincubated with recombinant human interleukin 1 β
25 or the phorbol ester, phorbol 12-myristate 13-acetate, in the presence or absence of the cyclooxygenase inhibitor, indomethacin. Interleukin 1 β or phorbol ester increased the levels of procollagenase (assayed after trypsin activation) and the labeling of several medium proteins
30 by cells incubated with [³⁵S]methionine, independent of prostaglandin synthesis. The labeling of 55 kD protein immunocomplexed with antibodies to procollagenase was also increased. The increased synthesis of procollagenase was paralleled by increased cellular
35 levels of procollagenase mRNA, determined with a cDNA

- 19 -

probe coding for human procollagenase. Thus the increased synthesis of procollagenase in response to the inflammatory mediator, interleukin 1, is controlled at a pretranslational level, possibly at the level of

5 transcription. The results are reported in Stephenson et al., Biochemical and Biophysical Research Communications 144:583-590, 1987), hereby incorporated by reference.

ii) Immortalized chondrocyte responses.

Both the C-20/A4 and T/C-28a2 immortalized
10 chondrocyte lines demonstrated responses to IL-1 similar to those that we demonstrated previously in normal human chondrocytes, including decreased expression of cartilage-specific matrix genes and increased expression of matrix metalloproteinase and immediate early genes.

15 The time courses of expression of the immediate early genes *egr-1*, *c-jun* and *Jun B*, the collagen genes $\alpha 1(\text{II})$ and $\alpha 1(\text{I})$ procollagens, and the metalloproteinase genes collagenase and stromelysin were monitored in the same experiments using primary cultures of human
20 chondrocytes, and the kinetics of their induction or suppression by IL-1 were compared. The C-20/A4 or T/C-28a2 chondrocytes were plated and grown for three days in culture medium containing 10% FCS. The cells were then made quiescent by serum starvation for 16-24 h in culture
25 medium containing 1% Nutridoma. IL-1 was then added in the absence or presence of the protein synthesis inhibitor cycloheximide (CHX; 10 $\mu\text{g/ml}$), the incubation continued for 24 hours and the cells harvested at various time points for extraction of total RNA and Northern blot
30 analysis.

Type II collagen mRNA was detected in both immortalized chondrocyte cultures and increased with time throughout the 24 h of incubation in 1% Nutridoma. At 1 h, IL-1 had no effect on type II collagen mRNA levels in
35 the C-20/A4 chondrocytes, but a slight suppression by IL-

- 20 -

1 was evident in the T/C-28a2 chondrocytes at both 1 and 6 h. A >60% suppression was achieved by 24 h. CHX could be observed to suppress type II collagen mRNA levels, particularly at 24 h, but it did not reverse or enhance the effect of IL-1. These results are consistent with previous experiments using normal human chondrocytes that indicated that the inhibitory effect of IL-1 on type II collagen synthesis was not a result of decreased $\alpha 1(\text{II})$ procollagen mRNA stability but suggested that cycloheximide may block the synthesis of an IL-1-induced repressor factor for $\alpha 1(\text{II})$ procollagen gene transcription. Type XI and IX collagen mRNAs showed similar appropriate expression patterns.

Addition of IL-1 to quiescent chondrocytes induced, within 1 h, high levels of *egr-1* and *jun B* (and *c-jun*)- mRNAs which declined rapidly thereafter (Yamin et al., J. Bone Mineral Res. 7:S120, 1992). Incubation with CHX augmented the levels of these mRNAs and prolonged the IL-1 induced expression past 6 h. The stimulation of *egr-1* mRNA by IL-1 appeared to be specific for normal as well as these immortalized chondrocytes, since serum, but not IL-1, induced *egr-1* mRNA in synovial and dermal fibroblasts and osteoblast-like cells, while IL-1 induced early transient expression of both *c-jun* and *jun B* mRNAs in all cell types studied thus far. Interestingly, *egr-1* mRNA was expressed in a somewhat reciprocal manner compared with type II collagen mRNA. This was particularly apparent in the T/C-28a2 cells that demonstrated low constitutive expression of *egr-1*. The C-20/A4 cells consistently expressed relatively high constitutive levels of *egr-1* mRNA at 1 h and lower levels of type II collagen mRNA compared with the T/C-28a2 cells. When the immortalized chondrocytes were incubated in the presence of FCS, however, the expression of type I collagen mRNA with little type II collagen mRNA was

- 21 -

avored. In addition, c-jun mRNA was detected and upregulated by IL-1, while egr-1 and jun B were barely detectable and only slightly upregulated in the presence of IL-1.

5 Constitutive levels of stromelysin mRNA were observed in both the C-20/A4 and T/C-28a2 chondrocytes. This expression was relatively stable at least up to 6 h, but induction by IL-1 was apparent by this time point. The inhibitory effect of CHX, however, on the induction
10 by IL-1 of the levels of collagenase and stromelysin mRNAs was not apparent until 24 h. In normal human chondrocytes constitutive levels of these metalloproteinase mRNAs are low or undetectable and the induction by IL-1 is fully blocked by CHX. No change in
15 GAPDH mRNA with time or in response to IL-1 was observable in these experiments.

E. Nuclear run-on transcription of the type II collagen gene is inhibited by IL-1 and IFN- γ in primary and immortalized human chondrocytes.

20 The nuclear run-off transcription assays were done as follows: Human immortalized costal chondrocytes were incubated for 42 h in the absence or presence of IL-1 β or IFN- γ in medium containing 10% FCS. The cells were washed in cold hypotonic buffer (20mM HEPES, pH 7.5, 5mM
25 KCl, 0.5 mM MgCl₂, 0.5 mM DTT) containing 0.2M sucrose, resuspended in hypotonic buffer containing no sucrose, placed on ice for 10 min and lysed with 5-8 strokes in a Dounce homogenizer. The resulting nuclear pellet after centrifugation at 500 X g for 5 min was resuspended in
30 storage buffer containing 40% glycerol, 50mM Tris-HCl, pH 8.0, 5 mM Mg acetate and 0.1mM EDTA and stored in 100 μ l aliquots containing 1 to 5 X 10⁷ nuclei at -70°C. The nuclei were then labeled once with α -³²P-UTP for 30 min at 30°C. The labeled RNA was extracted once with water-
35 saturated phenol-chloroform (2:1) and unincorporated

- 22 -

nucleotides were removed from the RNA by 2 sequential precipitations with 70% ethanol. Hybridizations were preformed at 42°C for 48 h using 1×10^6 cpm of labeled RNA and 2 μ g of each respective linearized plasmid DNA
5 immobilized on one strip of nitrocellulose per condition.

It has been reported previously that human recombinant preparations of either IL-1 β or IFN- γ could suppress the synthesis of type II collagen in cultured human adult articular or juvenile costal chondrocytes
10 (Goldring, M.B., et al., 1988, J. Clin. Invest., 82:2026-2037, Goldring, M.B., et al., 1986, J. Biol. Chem., 261:9049-9055). This suppression was associated with a decrease in the levels of α 1(II) procollagen mRNA. To determine whether this reduction in type II collagen mRNA
15 levels was due to decreased transcription of the type II collagen gene, nuclear run-on experiments were carried out on nuclei isolated from human costal chondrocytes after treatment with either IL-1 β or IFN- γ for 24 h. Nuclear run-off transcription assays demonstrated that
20 the suppressive effects of IL-1 β and IFN- γ on type II collagen gene expression are mediated primarily at the level of transcription. The signal of type II collagen gene transcription in chondrocytes at day 11 of culture was very strong compared with the other non-cartilage
25 collagen genes consistent with the relative levels of procollagen mRNAs that we reported previously (Goldring, M.B., et al., 1988, J. Clin. Invest., 82:2026-2037, Goldring, M.B., et al., 1986, J. Biol. Chem., 261:9049-9055). Labeled nascent transcripts from each incubation
30 condition were hybridized to separate strips of nitrocellulose containing 2 μ g of each unlabeled DNA encoding α 1 (II) procollagen, pBR325 plasmid and β -actin. IL-1 treatment of the chondrocytes resulted in a significant reduction in transcription of the type II
35 collagen gene (Figure 7). Preincubation of chondrocytes

- 23 -

with IL-1 in the presence of indomethacin potentiated this suppression consistent with effects we had shown previously on type II collagen synthesis and mRNA levels (Goldring, M.B., et al., 1988, J. Clin. Invest., 82:2026-2037, Goldring, M.B., et al., 1990, Eur. U. Rheumatol Inflamm., 10:10-21). The signals for transcription of the $\alpha 1(I)$ and $\alpha 2(I)$ procollagen genes were weak such that effects of IL-1 could not be assessed. Interestingly, the signal for $\alpha 1(III)$ procollagen gene transcription was strong and a stimulatory effect of IL-1 was observed.

IFN- γ also decreased transcription of the type II collagen gene (Figure 8A and 8B). This effect was somewhat dose-dependent with maximal (>95%) suppression at a relatively high concentration of 500 units per ml of IFN-V8.7 (Figure 8A). Transcription of the $\alpha 2(I)$ and $\alpha 2(III)$ procollagen genes was also inhibited by IFN- γ . Suppression by IFN- γ of transcription of the $\alpha 1(I)$ and $\alpha 1(II)$ procollagen genes was observed when the labeled transcripts were hybridized to the respective DNAs which has been run on agarose gels prior to blotting on nitrocellulose (Figure 8B). Hybridization against PBR325 plasmid DNA or a β -actin cDNA showed that these effects of IFN- γ were specific. Dot hybridization analysis of cytoplasmic RNAs extracted from parallel cultures demonstrated similar inhibitory effects of IL-1 β and IFN- γ on the levels of $\alpha 1(II)$ procollagen mRNA, as well as the effects of these cytokines on levels of $\alpha 1(I)$, $\alpha 2(I)$ and $\alpha(III)$ procollagen mRNAs that have been reported previously (Figure 9) (Goldring, M.B., et al., 1988, J. Clin. Invest., 82:2026-2037, Goldring, M.B., et al., 1986, J. Biol. Chem., 261:9049-9055).

F. Changes in mRNA stability do not account for the effects of IL-1 on the synthesis of type I or type II collagens by primary human chondrocytes.

- 24 -

Experiments with the protein synthesis inhibitor cycloheximide (CHX) and the mRNA-specific transcriptional inhibitor 5.6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), (Zandomeni, R. et al., 1986, J. Biol. Chem. 261:3414-3419), were carried out to determine whether the inhibitory effects of IL-1 on the expression of type II collagen mRNA are mediated at the level of mRNA stability. For these experiments, subconfluent chondrocytes were made quiescent by preincubation in the presence of 0.5% FCS for 12-24h. CHX (10 μ /ml) was then added in the absence or presence of IL-1 and/or indomethacin and cells were harvested for extraction of total RNA after 3, 6, and 24 hours of incubations. Northern blot analysis demonstrated that CHX reversed the IL-1-induced suppression of levels of α 1(II) procollagen mRNA at all three time points, but only partially at 24 h (Figure 10). This effect of CHX was also observed in another experiment in which DRB (10 μ g/ml) was added after 24 h of incubation with IL-1, indomethacin and/or CHX. Northern blot analysis of total RNAs harvested from cells after 5, 10 and 24 h of incubation with DRB demonstrated no change in the relative stability of the 5.5 kb type II collagen transcript under the different preincubation conditions (Figure 11). Similar effects of CHX and DRB have been observed in at least three separate experiments.

To determine whether protein synthesis is required for the IL-1-induced increase in the accumulation of α 1(I) procollagen mRNA levels, duplicate blots prepared for the experiments shown in Figures 10 and 11 were probed at the same time with the 32 P-labeled α 1(I) procollagen cDNA. CHX had no significant effect on the levels of type I collagen mRNA in the absence of IL-1 (Figure 10). Although no IL-1-induced increase in the level of α 1(I) procollagen mRNA was observed after 3 and

- 25 -

6 h of treatment with IL-1, coincubation with CHX increased the levels of $\alpha 1(I)$ procollagen mRNA above those observed with IL-1 alone. In contrast, the increase in the level of $\alpha 1(I)$ procollagen mRNA observed 5 after 24 h of treatment with IL-1, was inhibited by CHX. This effect of CHX on type I collagen mRNA was also observed at 24 h in another experiment shown in Figure 11. These effects of IL-1 and CHX, however, could not be accounted for by changes in type I collagen mRNA 10 stability, since there was no change in the slopes of the decay curves during incubation with DRB.

Indomethacin was added in these experiments because we showed previously that increased concentrations of PGE_2 prevent the stimulatory effect of 15 IL-1 on type I collagen gene expression. In the experiment shown in Figure 10, incubation of chondrocytes for 24 h with IL-1 increased the levels of type I collagen mRNA in either the absence or presence of indomethacin.. In the experiment shown in Figure 11, 20 however, the level of type I collagen mRNA was decreased after incubation for 24 h with IL-1 alone, but coincubation with indomethacin reversed this suppression and unmasked the stimulatory effect of IL-1. The apparent discrepancy between the two experiments may be 25 accounted for by the different concentrations of serum used. Although in both experiments, the cells were serum-starved by incubation in 0.5% FCS for 24 h prior to the addition of IL-1, indomethacin and CHX, the subsequent incubations were performed in 0.5% FCS (with 30 medium change) in the experiment shown in Figure 10 and in 1.0% FCS (with medium change) in the experiment in Figure 11. These differences in serum concentrations and medium renewal could result in differences in the availability of arachidonic acid substrate for 35 prostaglandin synthetase with low concentrations of PGE_2

- 26 -

produced in response to IL-1 under conditions of serum starvation.

G. Effects of IL-1 on the synthesis of type I or type II collagens by immortalized human chondrocytes:

5 Assay for mRNA stability.

The stability of type I and type II collagen mRNA's following IL-1 administration may be determined for immortalized chondrocytes. This determination may be made using the techniques described in Section (E),
10 above, on candidate cell lines.

H. Inhibition of expression of the type II collagen gene by IL-1 and IFN- γ in primary chondrocytes in transient expression experiments.

The complete DNA sequence of the 5' portion of the
15 human type II procollagen gene (COL2A1) has been published (Ryan, M.C., et al., 1990, *Genomics*, 8:41-48). Availability of the COL2A1 promoter/first intron construct (pCAT-B/4.0) has allowed examination of its expression for the first time in human chondrocytes.
20 This construct contains potential regulatory sequences that are homologous to silencer sequences responsive to fibroblast-specific nuclear factors in the upstream promoter region and a tissue-specific enhancer element in the first intron (Ryan, M.C., et al., 1990, *Genomics*,
25 8:41-48). We used the pCAT-B/4.0 construct to determine the conditions required for its expression in human chondrocytes and to determine whether its expression could be inhibited by IL-1 and IFN- γ .

The pCAT-B/4.0 plasmid construct was prepared in
30 the following manner: A plasmid containing 4.0 kb of DNA sequences of COL2A1, including -577 bp of upstream promoter sequence and 3428 bp downstream of the mRNA start site (242 bp of exon 1 and approximately 2/3 of the first intron), was constructed using the pCAT[™]-Basic
35 plasmid (Promega). This 4.0 kb Pst I fragment was cloned

- 27 -

into the PstI site located immediately upstream of the CAT gene and the plasmid was transfected into the JM109 bacterial strain and grown in the presence of ampicillin.

The transient expression assay was performed using
5 immortalized chondrocytes from the C-20/A4 line.
Immortalized chondrocytes were plated at 0.5×10^6 cells per 100-mm dish in Ham's F12/DMEM (1:1) containing 10% FCS and allowed to settle down for 24 hr. The medium was then changed and the cells transfected with 10 μ g of
10 plasmid DNA by the calcium-phosphate method (4h) followed by glycerol shock (2 min). After washing, the cells were allowed to recover in culture medium containing Ham's F12/DMEM/10% FCS. The medium was then changed to Ham's F12/DMEM containing 1% Nutridoma-SP (Boehringer-
15 Mannheim). IL-1 or IFN- γ were added after 16 hours of incubation in Ham's F12/DMEM/1% Nutridoma (Recovery experiments) or IL-1 β or IFN- γ were added immediately following glycerol shock, and washing, in the concentrations shown in Ham's F12/DMEM/1% Nutridoma (No
20 Recovery Experiments). The cultures were incubated for 24-48 h and harvested for CAT assay. Cell extracts were assayed for CAT activity by the fluor diffusion method (Neumann et al., Biotechniques (1987) 5:444 and Eastmann, Biotechniques (1987) 5:730). Data points for graphs were
25 selected from a time point on the linear part of the curve.

Monolayer cultures of chondrocytes isolated from two separate costal cartilage specimens (C-32 and C-33 at passage 2 and 1, respectively) were plated either on
30 agarose, as described by Castagnola et al. (Castagnola, P., et al., 1986, J. Cell Biol., 102:2310-2317), or tissue culture plastic in Ham's F12/DMEM containing 10% FCS and cultured for 2 weeks with weekly medium changes. The cells on agarose were then transferred to tissue
35 culture plastic and those on plastic were passaged to

- 28 -

clean dishes at 10^6 cells per 100-mm dish and allowed to settle down for 24h. The medium was then changed and the cells transfected with 10 μ g of plasmid DNA by the calcium-phosphate method (4h) followed by glycerol shock
5 (2 min). After washing, the cells were allowed to recover overnight in 10% FCS. Separate cultures were then incubated in culture medium containing 10% FCS, 1% Nutridoma-SP or 1% ITS+ without or with hIL- β (100 pg/ml) for 24 h and harvested for CAT assay.

10 The pCAT-B/4.0 plasmid did not express in chondrocytes incubated in the presence of 10% FCS, whether or not they had been passaged over agarose. The pCAT-B/4.0 plasmid did express in chondrocytes after
15 either agarose or monolayer culture in the presence of an insulin-containing serum substitute, particularly in 1% Nutridoma. Another insulin-containing serum substitute, ITS+, was also permissive for expression of pCAT-B/4.0 in chondrocytes but was more effective in cultures that had
20 been redifferentiated on agarose than in those that had been subcultured only on tissue culture plastic. All further transient expression assays were performed, therefore, in culture medium containing 1% Nutridoma.

The effects of recombinant preparations of human IL- β and IL- α at 50, 200 and 500 pg/ml on transient
25 expression of pCAT-B/4.0 were compared in chondrocytes (passage 2) that had been subcultured on tissue culture plastic. Both IL-1 β and IL-1 α inhibited COL2A1 expression in a dose-dependent manner. IL-1 β was more potent than IL- α , consistent with their relative effects
30 on type II collagen synthesis and levels of α 1(II) procollagen that we had observed previously (Figure 13). In addition, indomethacin potentiated the inhibitory effect of IL-1 β added at 50, 100 and 500 pg/ml (Figure 14).

- 29 -

The expression of pCAT-B/4.0 was tissue-specific but not species-specific, since it expressed in rat chondroblasts but not in human dermal fibroblasts. Moderate expression of pCAT-B/4.0 could be observed in the conditions, although expression was lower than in human chondrocytes; this expression was inhibited by human IL-1 β and by human IFN- γ (Figure 15). Human IL-1 α also was an effective inhibitor of pCAT-B/4.0 expression in the rat chondroblasts (data not shown). However, murine IFN- γ was used in the rat cultures, since IFN- γ is relatively species specific. No expression of pCAT-B/4.0 could be observed in human dermal fibroblasts (Figure 16). Furthermore, the pCAT-Control expressed in human fibroblasts (Figure 16), as well as in the rat chondroblasts (Figure 15) and human chondrocytes (not shown), but this expression was not inhibited by IL-1 and IFN- γ . These results indicate that IL-1- and IFN- γ -responsive elements reside in the 5'-flanking sequences of the COL2A1 rather than in the SV40 or plasmid DNA sequences in pCAT-B/4.0 construct.

When the 0.6 kb EcoR1/BamH1 fragment (+2680/+3370) was removed by reducing the construct to +2400 (pCAT-B/2.9), the expression in chondrocytes was reduced markedly (Figure 17). Virtually no expression was observed when the construct was reduced to +1400 (pCAT-B/2.0). Interestingly, IL-1 β inhibited the residual activity of the pCAT-B/2.9 construct. These results suggest that the negative regulatory element important for the response to IL-1 is not contained in the domain spanning +1400 to +3500, but is likely to be in the promoter region or possibly in the first part of intron 1.

I. Inhibition of expression of the type II collagen gene by IL-1 and IFN- γ in immortalized human chondrocyte transient expression experiments.

- 30 -

The C-20/A4 cell line responds to both IL-1 β and IFN- γ by suppressing the expression of the cartilage-specific type II collagen gene (COL2A1) when presented on a transiently transfected plasmid. This expression was monitored using a COL2A1-CAT vector. Figure 17B shows the expression from the pCAT-B/4.0 vector in response to the addition of either IL-1 β or IFN- γ . The bar graph at the left indicates the level of CAT in response to increasing concentrations of IL-1 β . The bar graph on the right indicates the level of CAT expression in response to IFN- γ . Recovery and no recovery conditions of the transient expression assay are described above. IL-1 β was obtained as described above. Recombinant preparations of human IFN- γ (2×10^6 or 2.65×10^7 units/mg) or mouse IFN- γ (17.4×10^6 or 5.2×10^7 units/mg) were obtained from Genentech, Inc. or they were purchased from AMGEN, Thousand Oaks, CA (hIFN- γ , 1×10^7 units/mg) or from Genzyme, Boston, MA (mIFN- γ , 9×10^6 units/mg).

Suppression of type II collagen production in chondrocytes in response to both IL-1 β and IFN- γ has been shown to occur in primary human chondrocyte cultures (Goldring et al., 1988, supra; Goldring et al., 1986, supra). In addition, IL-1 β has been shown to decrease type II collagen mRNA in intact cartilage (Tyler and Benton, 1988, Coll. Relat. Res. 8:393). Type II collagen is a cartilage specific gene and the suppression of type II collagen gene expression which is observed in chondrocytes in response to both IL-1 β and IFN- γ is a phenotype of fully differentiated chondrocytes. The finding provides a further demonstration of the fully differentiated phenotype of the C-20/A4 cell line. Similar results are obtained with the T/C-28a2 cell line.

- 31 -

J. Characterization of COL2A1 regulatory sequences that respond to IL-1-induced transcription factors in immortalized chondrocytes:

We showed previously that IL-1 suppresses transcription of the type II collagen gene (COL2A1) in human chondrocytes. To determine the DNA sequences that respond to IL-1, a plasmid containing various portions of the promoter, exon 1 and intron 1 of COL2A1 fused the chloramphenicol acetyl transferase (CAT) reporter gene were transfected in the immortalized chondrocyte lines C-20/A4 and T/C-28a2. High levels of expression of pCAT-B/4.0 with COL2A1 sequences spanning -577 to +3428 were observed in both lines in the presence of 1% Nutridoma-SP. This expression was inhibited by IL-1 by 50-80%. Deletion of the putative enhancer region markedly reduced activity, but the residual activity of the pCAT-B/2.9 (-577/+2368) was suppressed by IL-1. The promoter constructs pCAT-E/0.7 (-577/+63) and pCAT-E/0.2 (-131/+63) in the Promega pCAT-Enhancer vector exhibited higher levels of expression than pCAT-B/4.0 but did not respond to IL-1. The pCAT-P/0.5 construct (-577/-132 in the pCAT-Promoter vector) and a pCAT-B/0.7+1.1 construct (-577/+63 <->+2369/+3426) containing the putative COL2A1 enhancer also expressed at levels of 85% and 25%, respectively, of pCAT-B/4.0 expression and responded to IL-1. The pCAT-E/0.2 and pCAT-E-0.7 constructs, but none of the other COL2A1 constructs, expressed in human fibroblasts. Recent gel shift experiments have demonstrated activities in nuclear extracts from immortalized human chondrocytes that bind specifically to sequences within the 0.5 kb upstream promoter fragment and are modulated by IL-1 (Yamin et al., Arthritis Rheum. 35:349, 1992).

Other Embodiments

- 32 -

I. Use of immortalized chondrocytes as a source of transplantation tissue and/or as drug delivery vectors.

The immortalized chondrocytes of the invention may be used as a source of transplantation tissue to replace, restore, or reconstruct cartilage tissues in a patient. In addition, these cells may be transplanted for use as site-specific delivery vectors for polypeptides of therapeutic use, particularly for the delivery of those polypeptides useful in the treatment of cartilaginous diseases. Allogenic transplantation techniques may be performed using the methods and artificial matrices disclosed in Vacanti et al., United States Patent, SN 5,041,138, hereby incorporated by reference. Using these techniques allogenic cells may be transfected with one or more desirable nucleic acid sequences, and seeded and cultured into a shaped matrix, and implanted into the patient. This technique is particularly desirable in the patient suffering from a loss of cartilage mass. One skilled in the art will appreciate that other techniques for cartilage ingraftment can be used to achieve the transplantation. For example, direct injection and transplantation via a seeded collagen matrix may be used (Grant et al., United States Patent SN 4,846,835, hereby incorporated by reference).

II. Assay for antivascular agents:

The immortalized chondrocytes of the invention may be used for the isolation of inhibitors of vascularization. Such factors have pharmacological value as suppressors of tumor growth. Immortalized chondrocytes which will endure and divide for large periods of time in culture offer advantages over the standard methods of isolating such compounds from harvested primary chondrocytes. The method provided by Kuettner et al. in United States Patent SN 4,356,261 is an example of a technique which can be used to isolate

- 33 -

such substances from the chondrocytes of the current invention. One skilled in the art will appreciate that other techniques are available and may be used for this purpose.

5 III. Construction of a cell line with the
 phenotype of diseased cartilaginous tissues.

Model cell lines for the study of arthritis and other diseases of the cartilage may be constructed by the introduction of vectors which overexpress a preferred
10 protein or by the engineering of null mutations in genes where decreased expression is considered a possible cause of the pathological phenotype. These cell lines may be constructed using one of the immortalized cell lines in combination with known vectors and cloned genes. Such
15 cell lines may be used to elucidate which of those factors altered in various cartilaginous diseases are actually causative in nature. Goldring (1992, In: Cartilage Degradation: Basic Research and Clinical Implications, Eds: F. Woessiner and D. Howell, Marcel
20 Dekker, Inc.), Heinegard and Oldberg (1989, FASEB 3:2042-2051), Heinegard and Saxne (1991, British Journal of Rheumatology 30(suppl 1): 21-24), Hardingham et al. (1991, British Journal of Rheumatology 30(suppl 1): 32-37), Eyre et al. (1991, British Journal of Rheumatology
25 30(suppl 1): 10-15), and Murphy et al. (1991, British Journal of Rheumatology 30(suppl 1): 25-31), hereby incorporated by reference, describe the various phenotypes associated with cartilaginous diseases and the cloning and sequencing of the relevant genes and can be
30 used for guidance in choosing the appropriate test compounds and indicator assays.

The following are examples of useful alterations which can be made to the immortalized chondrocyte cell line for the study of rheumatoid arthritis: 1) vectors
35 which increase expression of IL-1, IL-2, IL-4, IL-5, IL-

- 34 -

6, TNF- α , TNF- β , IFN- α , IFN- β , and the colony stimulating factors GM-, M-, and G-CSF.

IV. Assay for pharmaceutically useful compounds for the treatment of diseases of the cartilaginous
5 tissues.

Goldring (1992, In: Cartilage Degradation: Basic Research and Clinical Implications, Eds: F. Woessiner and D. Howell, Marcel Dekker, Inc.), Heinegard and Oldberg (1989, FASEB 3:2042-2051), Heinegard and Saxne (1991, 10 British Journal of Rheumatology 30(suppl 1): 21-24), Hardingham et al. (1991, British Journal of Rheumatology 30(suppl 1): 32-37), Eyre et al. (1991, British Journal of Rheumatology 30(suppl 1): 10-15), and Murphy et al. (1991, British Journal of Rheumatology 30(suppl 1): 25- 15 31) describe the known gene regulation and phenotypic phenomenon displayed in tissues affected by a variety of cartilaginous diseases. The cells of the invention may be used to screen for synthetic compounds or naturally occurring peptides which mitigate these pathological 20 events. Such compounds may be screened for and detected by applying the compounds of interest to immortalized chondrocytes in culture and monitoring the effects such compounds on gene regulation and protein synthesis using the probes described above in Section IIa of the 25 Preferred Embodiments. When applied to the immortalized chondrocytes of the invention (either those cells described in section II of the Preferred Embodiments or those cells further altered by the addition of nucleic acids as described immediately above in Section III of 30 the Other Embodiments) the useful pharmaceutical compound is one which alters the gene expression or protein production in a manner which mitigates one or more of the pathological phenotypes of a cartilaginous disease.

- 35 -

Table 1

<u>-Cartilage-specific phenotype:</u>		<u>T/C-28</u>	<u>C-20/A4</u>	<u>Primary</u>
5	-Type II, IX, and XI collagen mRNAs	+	+	+
	-PG-I and PG-II RNAs	+	+	+
	-Protein	+	+	+
	-SDS-PAGE (collagen II)	+	+	+
	-Immunocytochemistry (collagen II & PGs)	+	+	+
	-pCAT-B/4.0 expression	+	+	+
<u>-Responsiveness to IL-1:</u>				
15	-↓ type II collagen mRNA.	+	+	+
	-↑ type I collagen mRNA.	+	+	+
	-↑ collagenase mRNA	+	+	+
	-↑ stromelysin mRNA.	+	+	+
	-↑ jun-B, c-jun, egr-1 mRNAs	+	+	+

What is claimed is:

- 36 -

1. An immortalized human chondrocyte cell line having a homogeneous polygonal morphology and further characterized in that it

- a) expresses type II collagen
- 5 b) suppresses the production of type II collagen in response to IL-1 and IFN- γ ,
- c) expresses collagenase in response to IL-1, and
- d) secretes at least two of proteoglycans secreted by primary human chondrocytes, and
- 10 e) does not express the non-specific proteoglycan Versican.

2. A composition comprising an immortalized chondrocyte cell line in insulin-containing serum-free medium, said cell line being characterized in that it

- 15 a) expresses type II collagen
- b) suppresses the production of type II collagen in response to IL-1 and IFN- γ , and
- c) expresses collagenase in response to IL-1.

3. The composition of claim 2 wherein said
20 immortalized chondrocyte cell line secretes at least two of the proteoglycans secreted by primary human chondrocytes, and said cell line does not secrete proteoglycans detectable by Versican.

4. The immortalized cell line of claim 1,
25 derived from human primary chondrocytes transfected with origin defective SV40.

5. A method of producing and maintaining the immortalized human chondrocyte cell line of claim 1 comprising

- 30 a) providing human chondrocytes,

- 37 -

b) transfecting the chondrocytes with origin-defective SV40,

c) subculturing the transfected cells, and

d) selecting a cell line that displays a

5 cobblestone morphology and is further characterized in that it

1) expresses type II collagen

2) suppresses the production of type II collagen in response to IL-1 and IFN- γ ,

10 3) expresses collagenase in response to IL-1,

4) secretes a proteoglycan profile corresponding to that of mature human chondrocytes, and

e) maintaining said cell line in insulin-containing serum-free medium.

15 6. A method of treating a degenerative cartilage disease said method comprising a) providing cells obtained from the cell line of claim 1 wherein said cells are transfected with DNA encoding a therapeutic protein, and b) administering said transfected cells to said
20 patient.

7. The method of claim 5 in which said degenerative cartilage disease is osteoarthritis.

8. The method of claim 5 in which said cells are transfected with a gene encoding a cytokine inhibitor.

25 9. The method of claim 8 in which said cytokine is TGF- β .

10. The method of claim 8 in which said cytokine is IGF-I.

- 38 -

11. The method of claim 6 in which said cells are transfected with DNA encoding a cytokine.

12. The method of claim 11 in which said cytokine inhibitor is IL-1.

5 13. The method of claim 12 in which said cytokine inhibitor is an IL-1 receptor binding antagonist.

14. The method of claim 6 in which said cells are transfected with DNA encoding a protease.

15 15. The method of claim 6 in which said cells are transfected with DNA encoding a protease inhibitor.

16. The method of claim 6 in which said cells are transfected with DNA encoding an extracellular matrix component.

17. The method of claim 6 in which said
15 administering is done by localized injection.

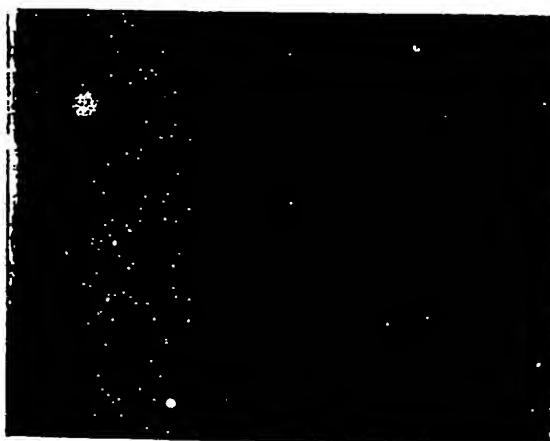
18. A method of harvesting an antivascular compound, said method comprising culturing the cells of claim 1 and purifying from said cells a compound which inhibits vascularization.

20 19. A method of screening compounds, said method comprising applying said compounds to the cells of claim 1 and monitoring said cells for alterations in gene regulation.

FIG. 1a



FIG. 1b



Type II collagen mRNA in C20/A-4

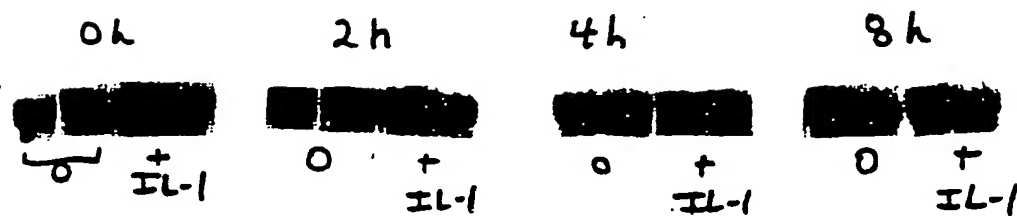
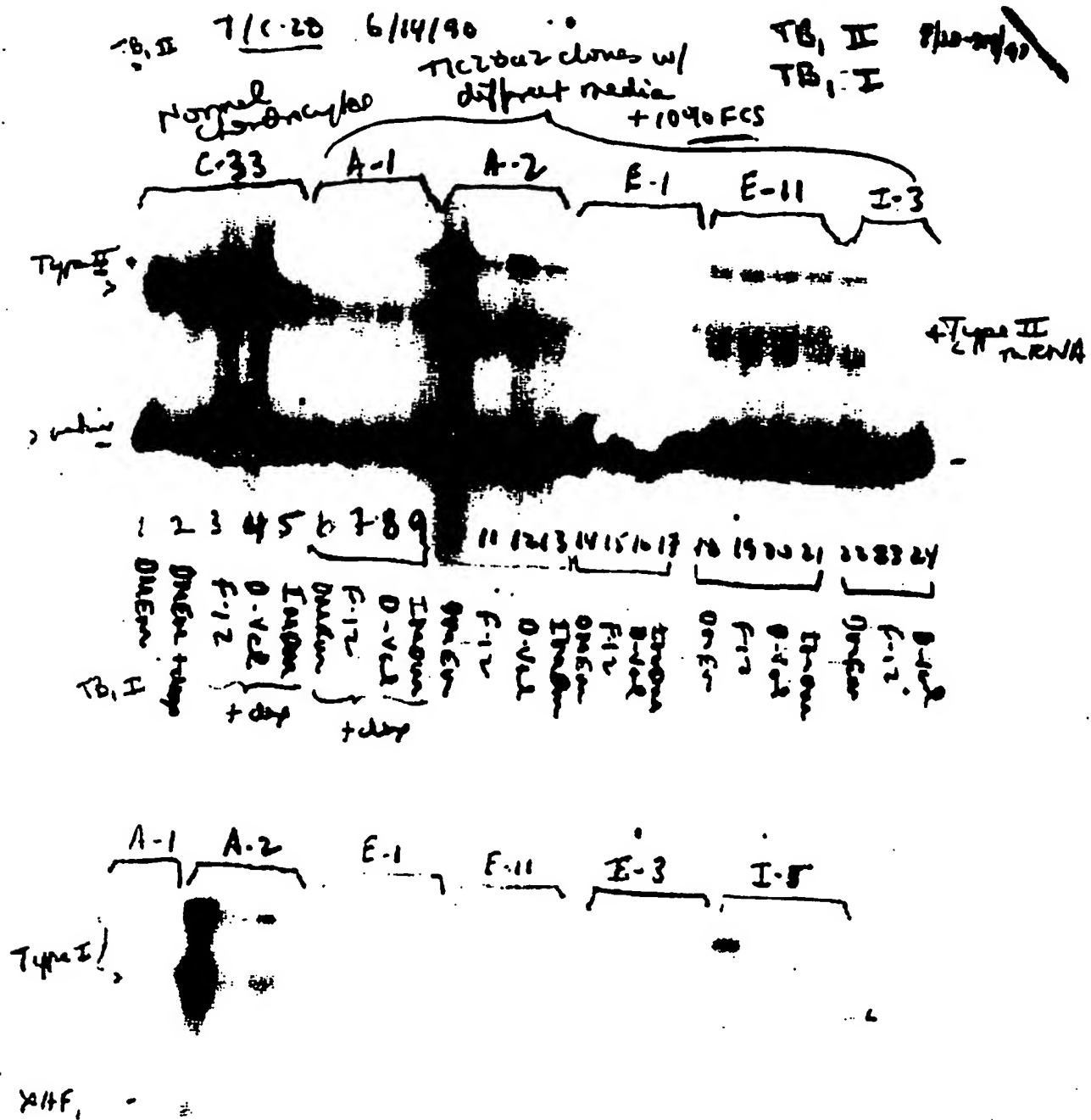


FIG. 2



Over ev, and plot II

TB,



← Type II
MCM

38, 29

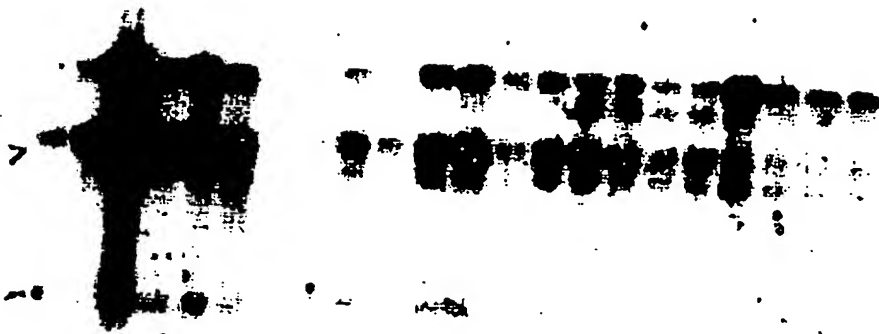


FIG. 3b

[illegible]

TBI II	Net- AGGRECAN	Human PGI	PGII	al(IX)
C33				
DMEM	+	+++	+	+
DMEM+ DEX				
F12	++	+	+	+
D-VAL?	+++	+++	+++	+
IMDM	++++	+++	+++	+
A1	+	++	+	+
DMEM				
F12				
D-VAL				
IMDM				
A2				
DMEM				
F12			+	+
D-VAL				+
IMDM				+
E1				+
DMEM				
F12				
D-VAL				
IMDM				
E11				
DMEM				
F12				+
D-VAL				+
IMDM				+
I3				+
DMEM				+
F12				+
D-VAL				+
IMDM				+
I3				+
DMEM				+
F12				+
D-VAL				+

Die folgenden Aufgaben sind zu lösen:

The 11:25 change we wanted for the
note was of change in price in
Brown Goldfishes & in the

FIG. 4

5/12

Human Costal Chondrocyte mRNAs

Day 9 of Culture

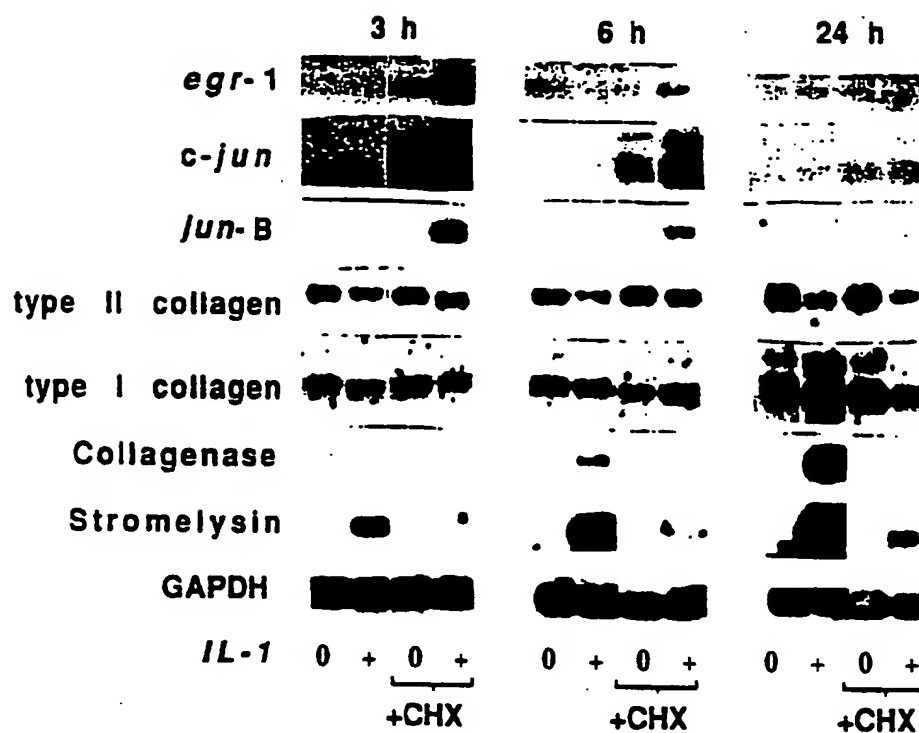


FIG. 5

c-Jun mRNA in C20/A4

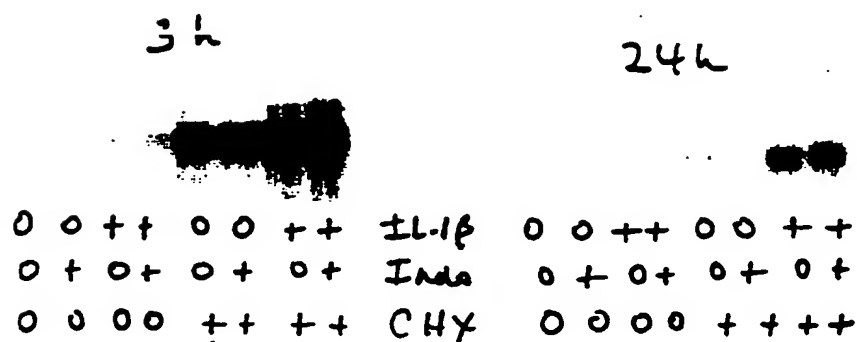


FIG. 6

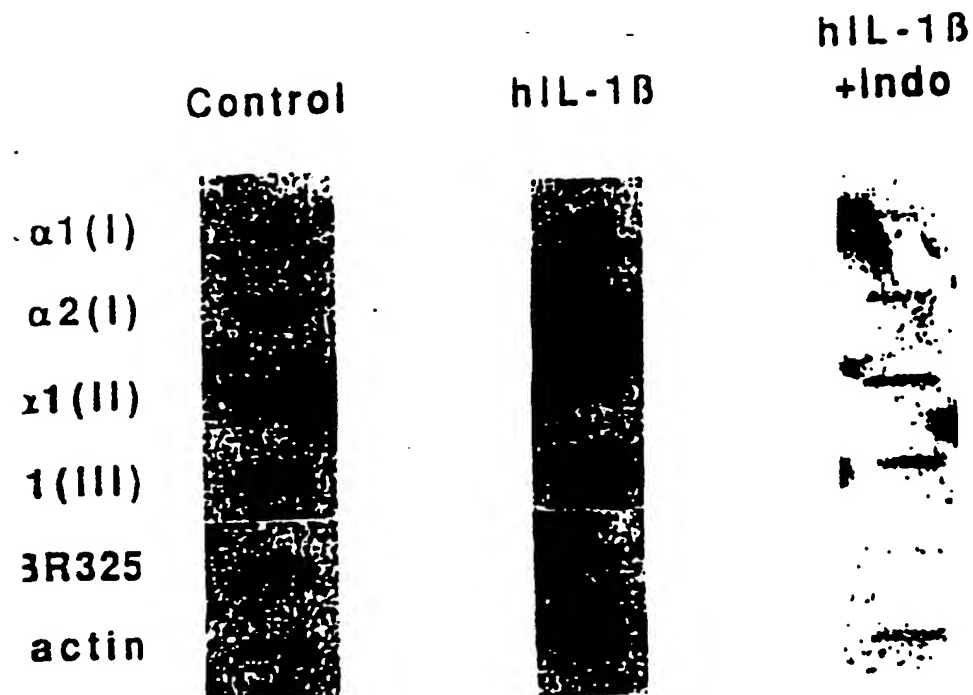


FIG. 7

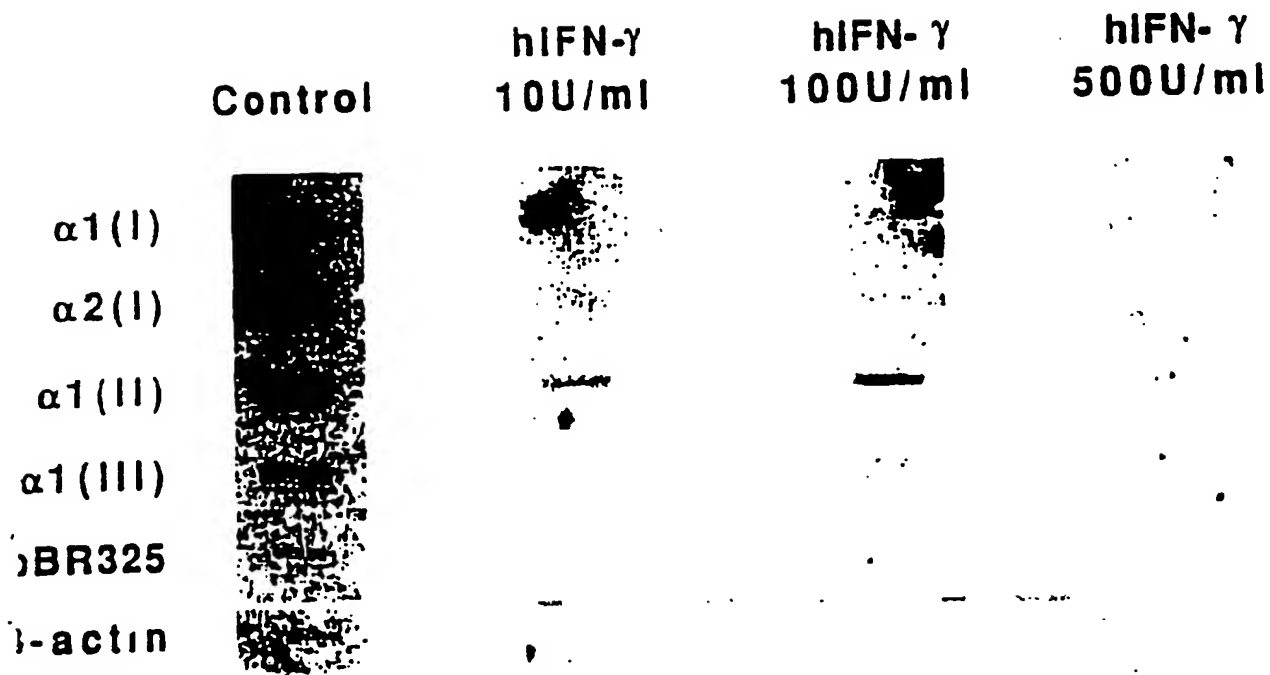


FIG. 8a

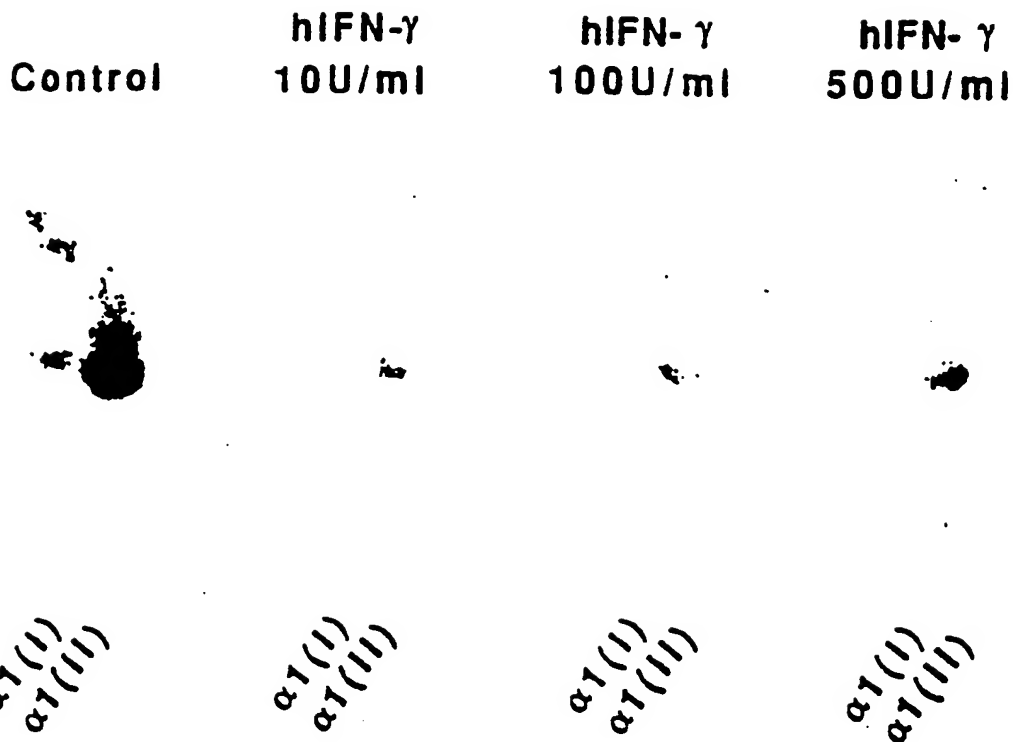


FIG. 8b

Procollagen mRNAs

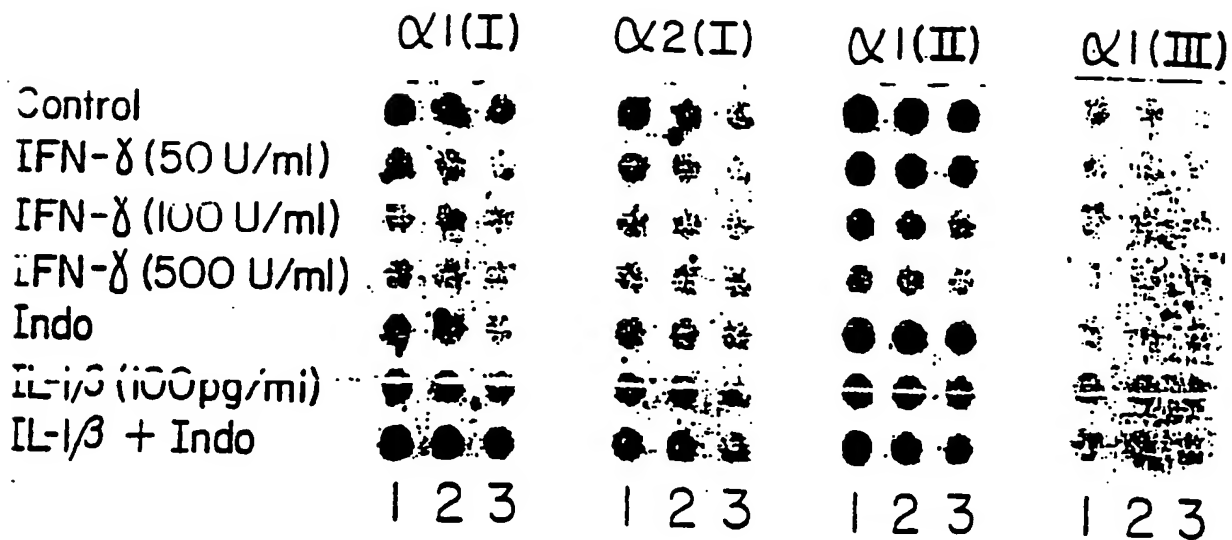


FIG. 9

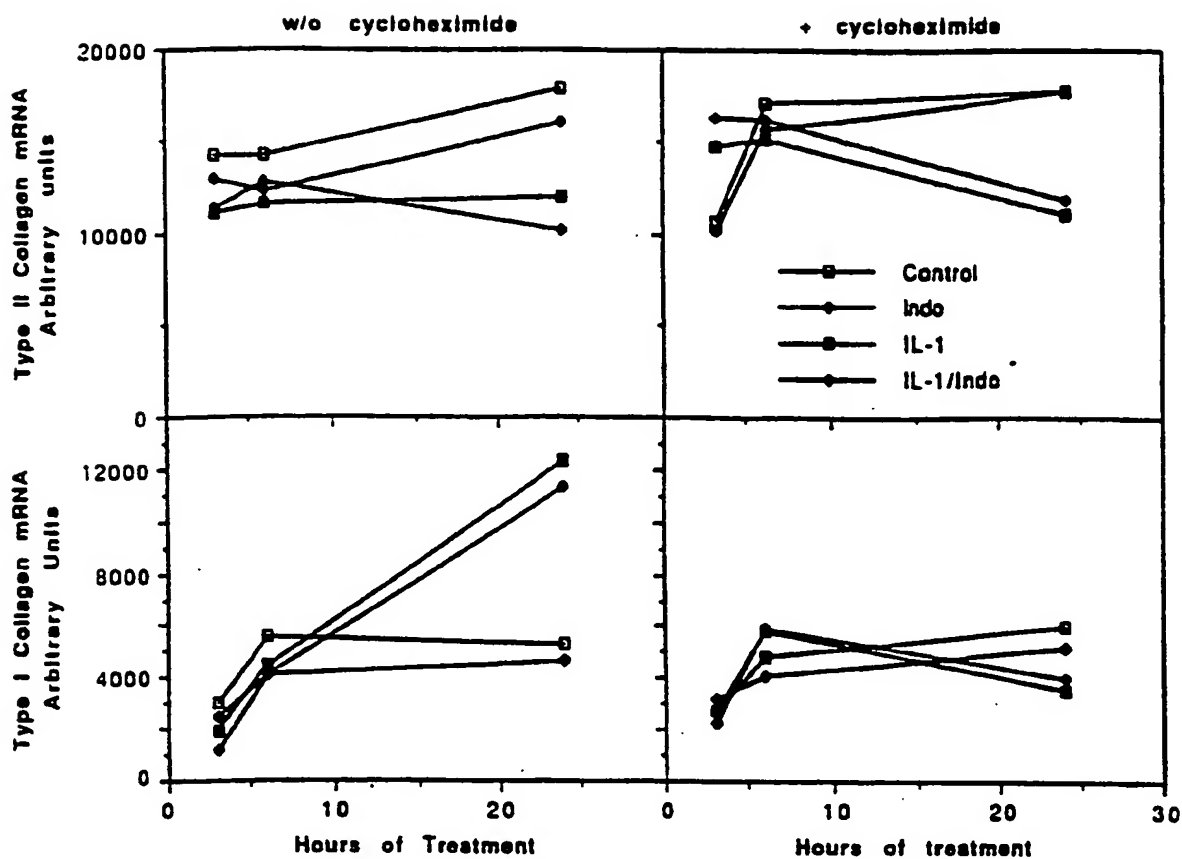


FIG. 10

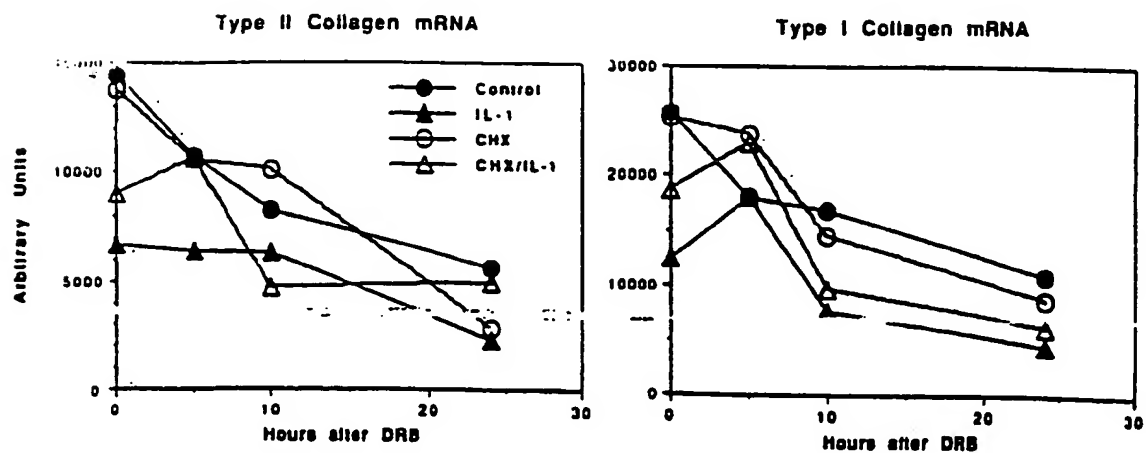


FIG. 11

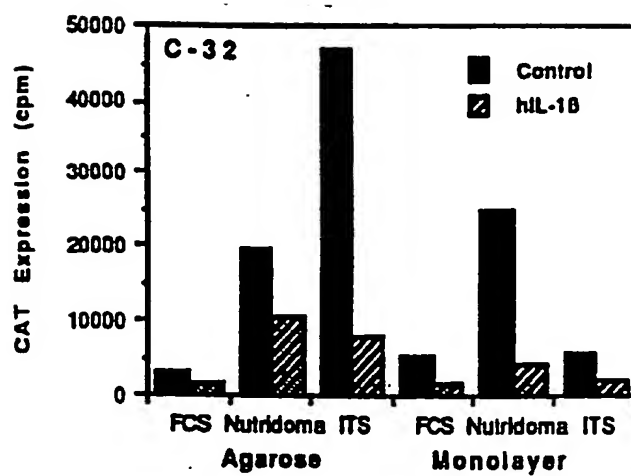


FIG. 12a

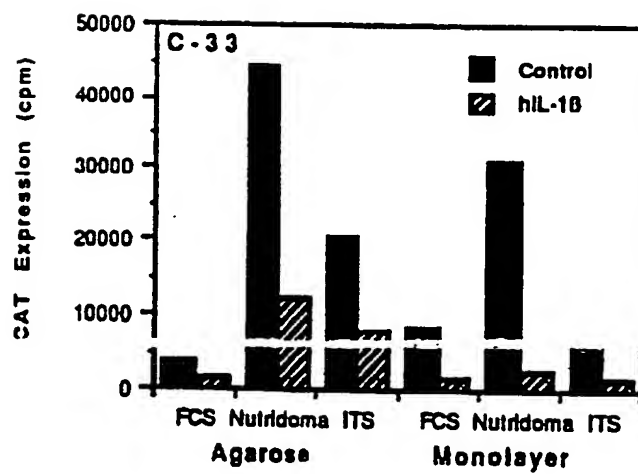


FIG. 12b

10/12

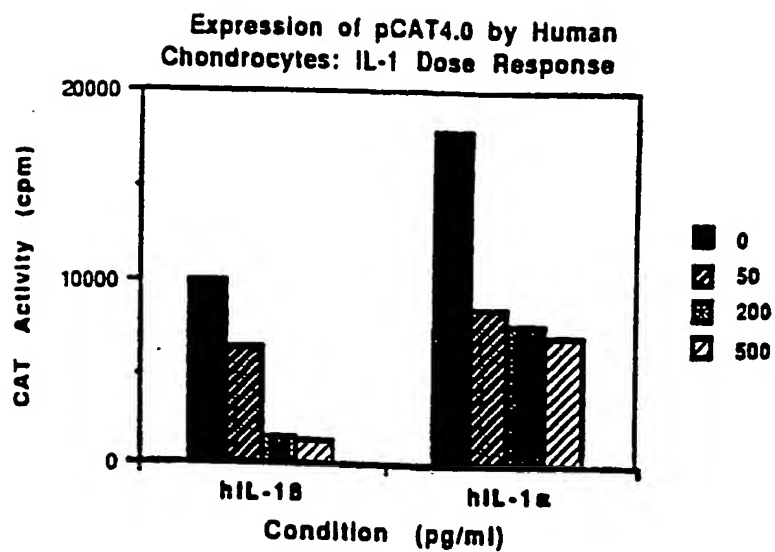


FIG. 13

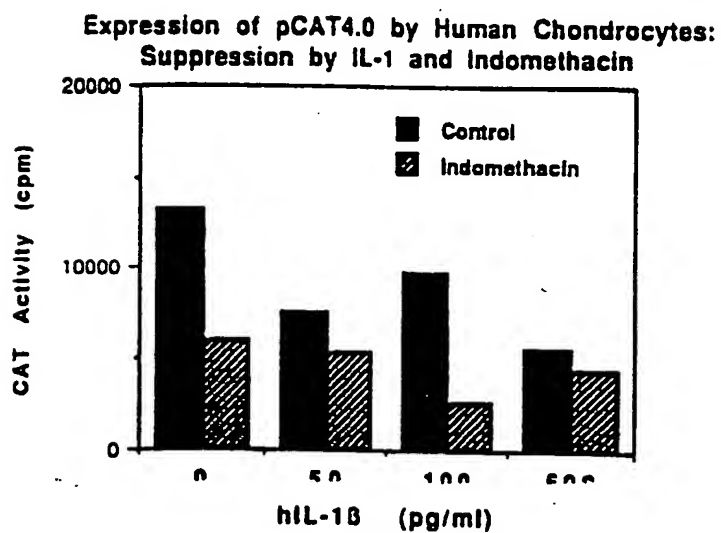


FIG. 14

11/12

Expression of pCAT4.0 by Rat Chondroblasts

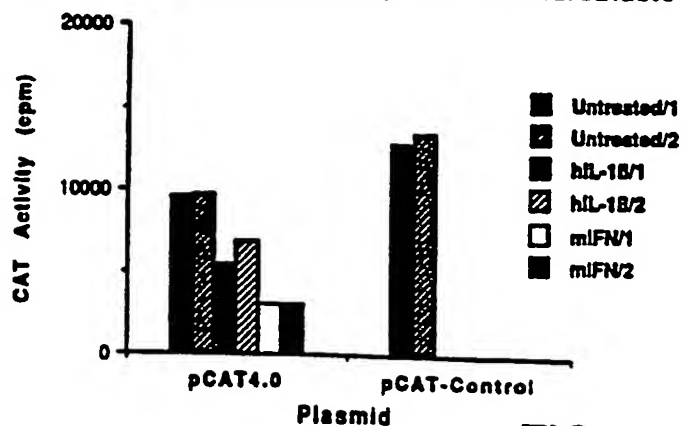


FIG. 15

Expression of pCAT4.0 by Human Fibroblasts

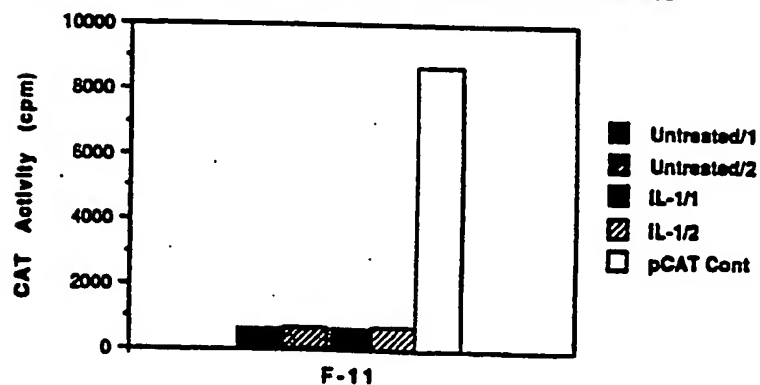


FIG. 16

Human Chondrocytes

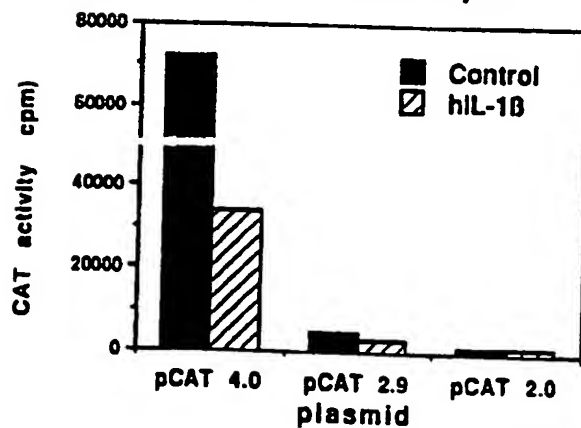


FIG. 17a

12/12

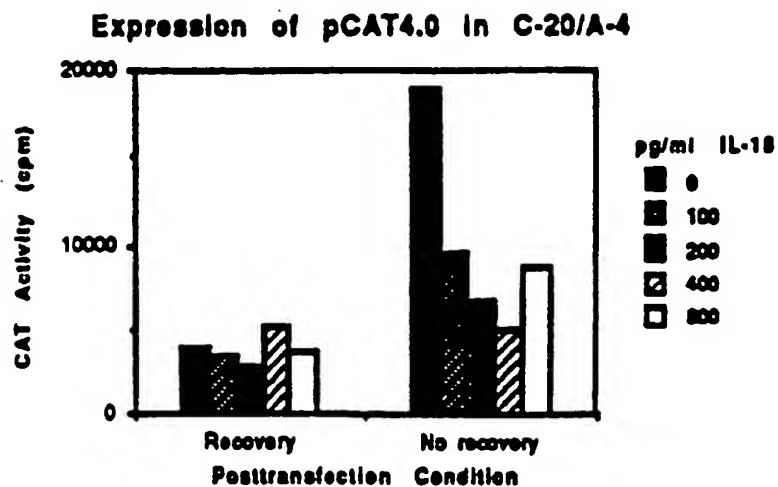


FIG. 17b

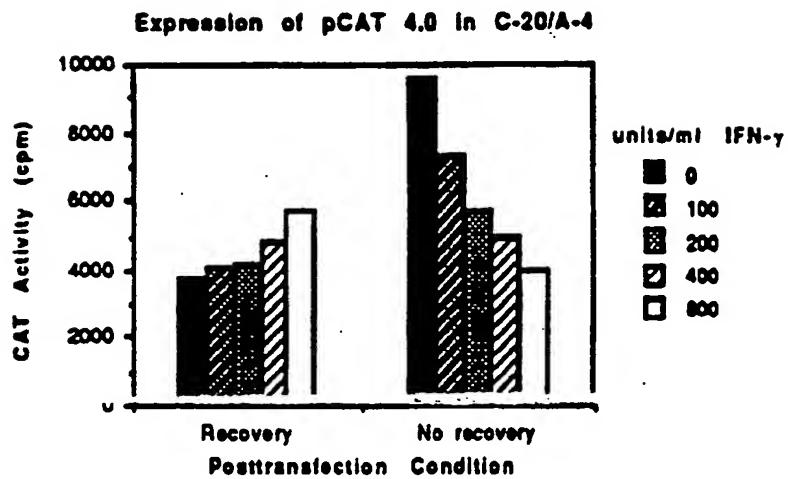


FIG. 17c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09718

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 5/06, 5/10; A61K 48/00; C12Q 1/02

US CL :435/240.21, 240.2, 172.3, 29; 424/93A

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.21, 240.2, 172.3, 29; 424/93A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

Search Terms: chondrocyte, immortalized, collagen, IL-1, TGF- β , IGF, cartilage degeneration, differentiation**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,707,448 (Major) 17 November 1987, see Column 6, line 7 to Column 8, line 32.	1-19
X	Cancer Research, Volume 49, issued 15 July 1989, Takigawa et al., "Establishment of a Clonal Human Chondrosarcoma Cell Line with Cartilage Phenotypes," pages 3996-4002, see page 3999, Table 1 and second column, line 5 to page 4000, first column, line 8; and Figure 7 on page 4000.	1

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	
A document defining the general state of the art which is not considered to be part of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

20 December 1993

Date of mailing of the international search report

JAN 03 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

GEORGE C. ELLIOTT

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09718

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Experimental Cell Research, Volume 178, issued 1988, Horton et al. "An Established Rat Cell Line Expressing Chondrocyte Properties," pages 457-468, see especially page 457, first line of text to page 458, line 24; page 459, last paragraph and page 461, first paragraph.	1-19
Y	Journal of Bone and Mineral Research, Volume 5, issued 1990 (abstract presented 28 August 1990), Apperly et al., "Immortalization of Human Osteoblast-Like Cells with SV-40 Large T Antigen," page S93.	1-19
Y	Experimental Cell Research, Volume 155, issued 1984, Adolphe et al., "Cell Multiplication and Type II Collagen Production by Rabbit Articular Chondrocytes Cultivated in a Defined Medium," pages 527-536, see entire document.	1-19
Y	Biochemical and Biophysical Research Communications, Volume 144, No. 2, issued 29 April 1987, Stephenson et al., "Stimulation of Procollagenase Synthesis Parallels Increases in Cellular Procollagenase mRNA in Human Articular Chondrocytes Exposed to Recombinant Interleukin 1 β or Phorbol Ester," pages 583-590, see entire document.	1-19
Y	The Journal of Biological Chemistry, Volume 261, No. 19, issued 05 July 1986, Goldring et al., "Immune Interferon Suppresses Levels of Procollagen mRNA and Type II Collagen Synthesis in Cultured Human Articular and Costal Chondrocytes," pages 9049-9056, see entire document.	1-19
Y	The Journal of Biological Chemistry, Volume 262, No. 34, issued 05 December 1987, Goldring et al., "Modulation by Recombinant Interleukin 1 of Synthesis of Types I and III Collagens and Associated Procollagen mRNA Levels in Cultured Human Cells," pages 16724-16729, see entire document.	1-19
Y	Journal of Clinical Investigation, Volume 82, issued December 1988, Goldring et al., "Interleukin 1 Suppresses Expression of Cartilage-Specific Types II and IX Collagens and Increases Types I and III Collagens in Human Chondrocytes," pages 2026-2037, see entire document.	1-19
Y	The New England Journal of Medicine, Volume 322, No. 18, issued 03 May 1990, Harris, Jr., "Rheumatoid Arthritis: Pathophysiology and Implications for Therapy," pages 1277-1289, see especially page 1286, lines 47-55.	6-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/09718

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Hematology Reviews, Volume 2, issued 1987, Williams et al., "Molecular Techniques in Hematopoiesis: Retroviral-Mediated Gene Transfer into Hematopoietic Stem Cells," pages 1-12, see entire document.	6-17